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High Population Genetic Diversity and Regional Differentiation in the Endemic *Vincetoxicum katoi–V. yamanakae* Complex (Apocynaceae; Asclepiadoideae) in Japan.

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We examined morphological differentiation and allozyme diversity in nine populations of *Vincetoxicum katoi* and its close relative *V. yamanakae*. Although, *V. yamanakae* has been distinguished from *V. katoi* in having a larger corolla and gynostegium, the principal component analysis based on 12 floral and three leaf characters did not support the distinction of the two taxa. The six allozyme loci examined revealed that population of *V. katoi* and *V. yamanakae* maintained high genetic diversity ($P = 72.1$, $A = 2.40$, $h = 0.313$). The nine populations were clustered into three regions (Kanto–Tokai; middle Kiniki; and Kii–Shikoku) based on genetic differences, contradicting the current taxonomic treatment. Considering the evidence, it is appropriate to treat *V. yamanakae* as a synonym of *V. katoi*. The high genetic differentiation among regions suggested that disjunct distribution of the *V. katoi–V. yamanakae* complex might reflect the persistence of refugia since the last glacial period. In particular, the middle Kinki (Hyogo) population is located outside of known evergreen forest refugia, suggesting that it might have survived during the glacial period within cooler vegetative zones, such as in temperate forests, whereas the populations on the Pacific-side retreated to warm-temperature coastal refugia.

Key words: allozyme, disjunct distribution, floral morophology, *Vincetoxicum katoi*, *Vincetoxicum yamanakae*

The Japanese flora is affected not only by the current climate but also by past geographic events (Aoki *et al.* 2006). Recent phylogeographic studies of plants in alpine-sub alpine regions (e.g., Fujii & Senni 2006, Ikeda *et al.* 2008), temperate forests (e.g., Hiraoka & Tomaru 2009, Kikuchi *et al.* 2010), and evergreen forests (e.g., Aoki *et al.* 2004, 2006) have provided new insights into the location of glacial refugia and the routes of postglacial range expansion of plants within the Japanese Archipelago. Palynological records for lowland evergreen forests in Japan have suggested the existence of multiple refugia along the Pacific coast during the last glacial period (Kamei and Research Group for Biogeography from Würm Glacial 1981, Hattori 2002). Using chloroplast DNA sequence variations, Aoki *et al.* (2004, 2006) confirmed the existence of refugia for several evergreen tree species outside of Kyusyu. The locations of some refugia, however, were only weakly confirmed by molecular-based analyses.

Disjunct distributions, the presence of plants or animals occurring in two or more remote geographic areas, are thought to be caused by three major processes (Raven 1972, Cox & Moore 2005, Lomolino *et al.* 2005): 1) ancient tectonic events such as continental drift that resulted in intercontinental splits in species distributions, 2) more recent climate changes (the glacial-interglacial cycle during the Quaternary), and 3) long-distance dispersal. Alpine plants, which exhibit island-like disjunct distributions, have been the subject of intensive research on glacial refugia using molecular phylogenetic approaches (e.g., Fujii & Senni 2006). As in alpine regions, lowland plants with disjunct distributions are expected to provide information regarding drastic distribution changes during glacial periods. This is because even though lowlands have been affected by long-term deforestation, their current disjunct distributions may represent footprints of glacial refugia.

Vincetoxicum katoi (Ohwi) Kitagawa is an endemic perennial understory herb that occurs chiefly in *Cryptomeria japonica* (L. fil.) D. Don plantations, and is disjunctly distributed from lowlands to hills on the Pacific side of the Kanto district and in the southern and middle parts of the Kinki (Kii peninsula and Hyogo) district on Honshu, Japan (Fig. 1) (Yamazaki 1993). *Vincetoxicum yamanakae* (Ohashi & Ohwi) Ohashi is closely related to *V. katoi*, and also occurs chiefly in *Cryptomeria japonica* plantations in the lowlands of Shikoku (Fig. 1) (Yamazaki 1993). *V. yamanakae* is distinguished from *V. katoi* by having

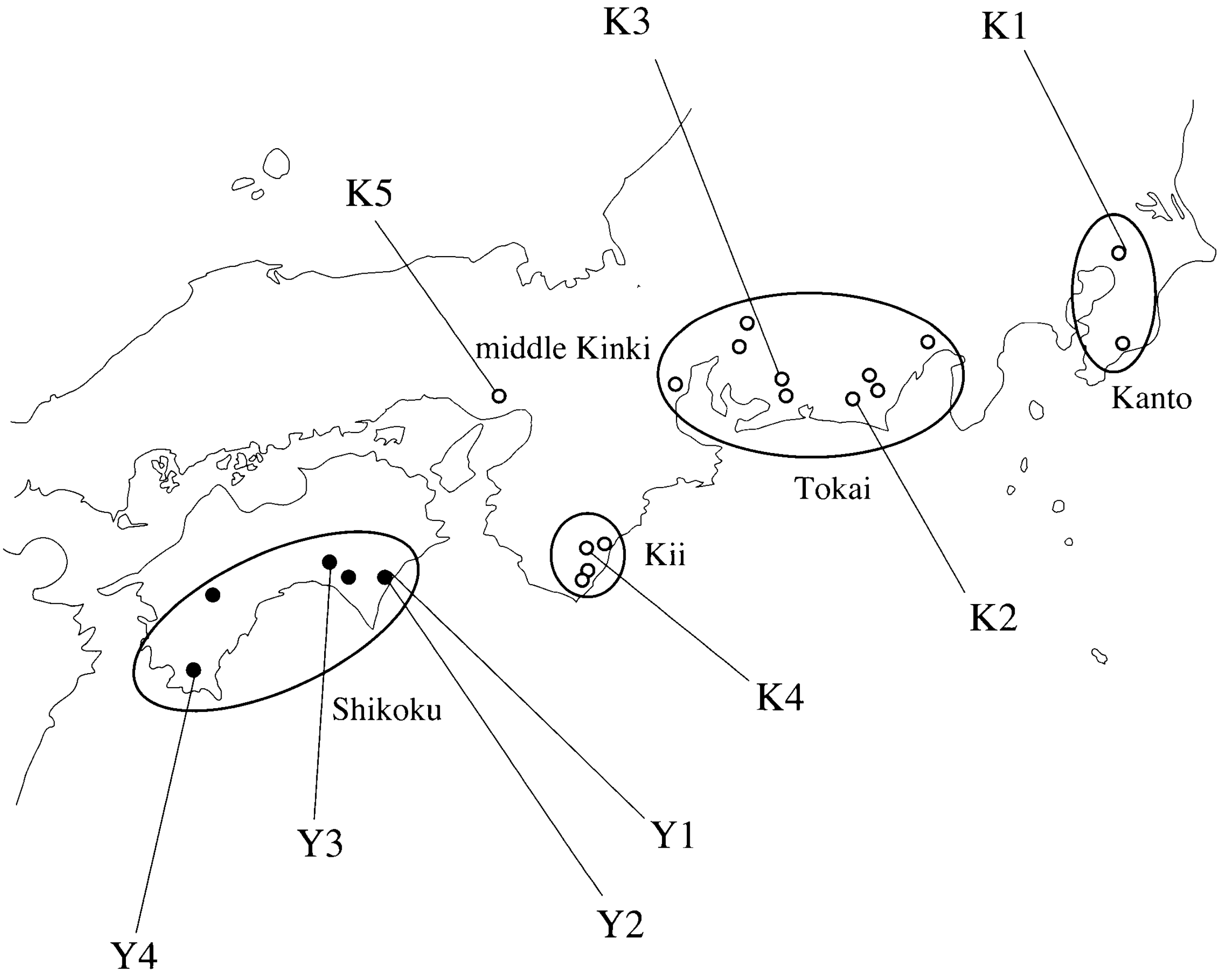

Fig. 1. Geographic distribution of the *V. katoi* (open circle)–*V. yamanakae* (closed circle) complex based on herbarium specimen information and location of sampling sites. For population codes, see Table 1.

a larger corolla and gynostegium (Yamazaki 1993). Currently, *V. katoi* is listed as 'vulnerable', whereas *V. yamanakae* is listed as 'critically endangered' because of its more restricted distribution (Environment Agency of Japan 2007). As suggested by Yamazaki (1993), however, the corolla size in species of asclepiads is variable. Ambiguity in taxonomic status may lead to erroneous conservation decisions and designations (Frankham *et al.* 2004). To assign the proper conservation designation to species such as *V. katoi* and *V. yamanakae*, their morphological and genetic distinction should be determined.

The first objective of this study was to ascertain the distinction between *Vincetoxicum katoi* and *V. yamanakae* by quantitatively evaluating their morphological traits. The second objective was to assess the genetic diversity and differentiation of populations of *V. katoi* and *V. yamanakae* using allozymes. We also discuss the factors that influence the current disjunct distribution of the populations of *V. katoi* and *V. yamanakae.*

Materials and Methods

Plant materials

Nine populations of *Vincetoxicum katoi* and *V. yamanakae* (Fig. 1, Table 1) were sampled and tentatively classified as either *V. katoi* (designated K) or *V. yamanakae* (designated Y) following Yamazaki's (1993) report of their geographical distributions. Five populations from Kanto, Tokai, Kii, and middle Kinki were classified as *V. katoi*, and four populations from Shikoku were classified as *V. yamanakae*. A combination of inflorescences and leaves was collected from flowering individuals. Inflorescences were fixed in FAA (a mixture of formalin, glacial acetic acid, and 70% ethanol at a ratio of 5:5:90 by volume) for measurement of floral traits. Leaves were transported on ice to the laboratory and refrigerated (4°C) for several days until being extracted for electrophoresis analysis. Voucher specimens representing each population were deposited in the Herbarium of Tohoku University (TUS), Shoei Junior College (SHO) and Tokushima Prefectural Museum (TKPM).

Morphology

Morphological measurements were recorded for 72 and 90 individuals from four populations each of *Vincetoxicum katoi* and *V. yamanakae*, respectively (Table 1). A digital caliper and a micrometer under a dissecting microscope were used to measure 12 floral features: pedicel length (PL), corolla diameter (CLD), corolla lobe length and width (CLL and CLW, respectively), corona diameter (CND), length of corona lobe (CNL), diameter and length of the gynostegium (GYD and GYL, respectively), length and width of the corpusculum (COL and COW, respectively), and length and width of the pollinia (POL and POW, respectively) (Fig. 2). For leaf samples, three

TABLE 1. Population code, localities, altitude and sample size for morphological (N_M) and allozyme (N_A) analysis for the populations examined in the *V. katoi–V. yamanakae* complex.

Population code	Locality	Altitude	N_M	N_A	Voucher*
K1	Chiba Pref., Tomisato	40 m	25	24	*T. Y. & A. Y. 7416* (TUS)
K2	Shizuoka Pref., Fukuroi	40 m	-	13	*T. Y. & A. Y. 7181* (TUS)
K3	Aichi Pref., Shinshiro	200 m	19	31	*T. Y. & A. Y. 7421* (TUS)
K4	Wakayama Pref., Shingu	60 m	8	6	*T. Y. & A. Y. 11169* (TKPM)
K5	Hyogo Pref., Kobe	600 m	20	25	*T. K. 28983* (SHO)
Y1	Tokushima Pref., Kaiyo	60 m	24	24	*T. Y. 11175* (TKPM)
Y2	Tokushima Pref., Kaiyo	70 m	23	27	*T. Y. 11183* (TKPM)
Y3	Kochi Pref., Konan	40 m	25	51	*T. Y. & A. Y. 7438* (TUS)
Y4	Kochi Pref., Tosashimizu	140 m	18	25	*T. Y. & A. Y. 7198* (TUS)

* T. Y. ; Yamashiro, T.; A. Y. ; Yamashiro, A.; T. K.; Kobayashi, T.

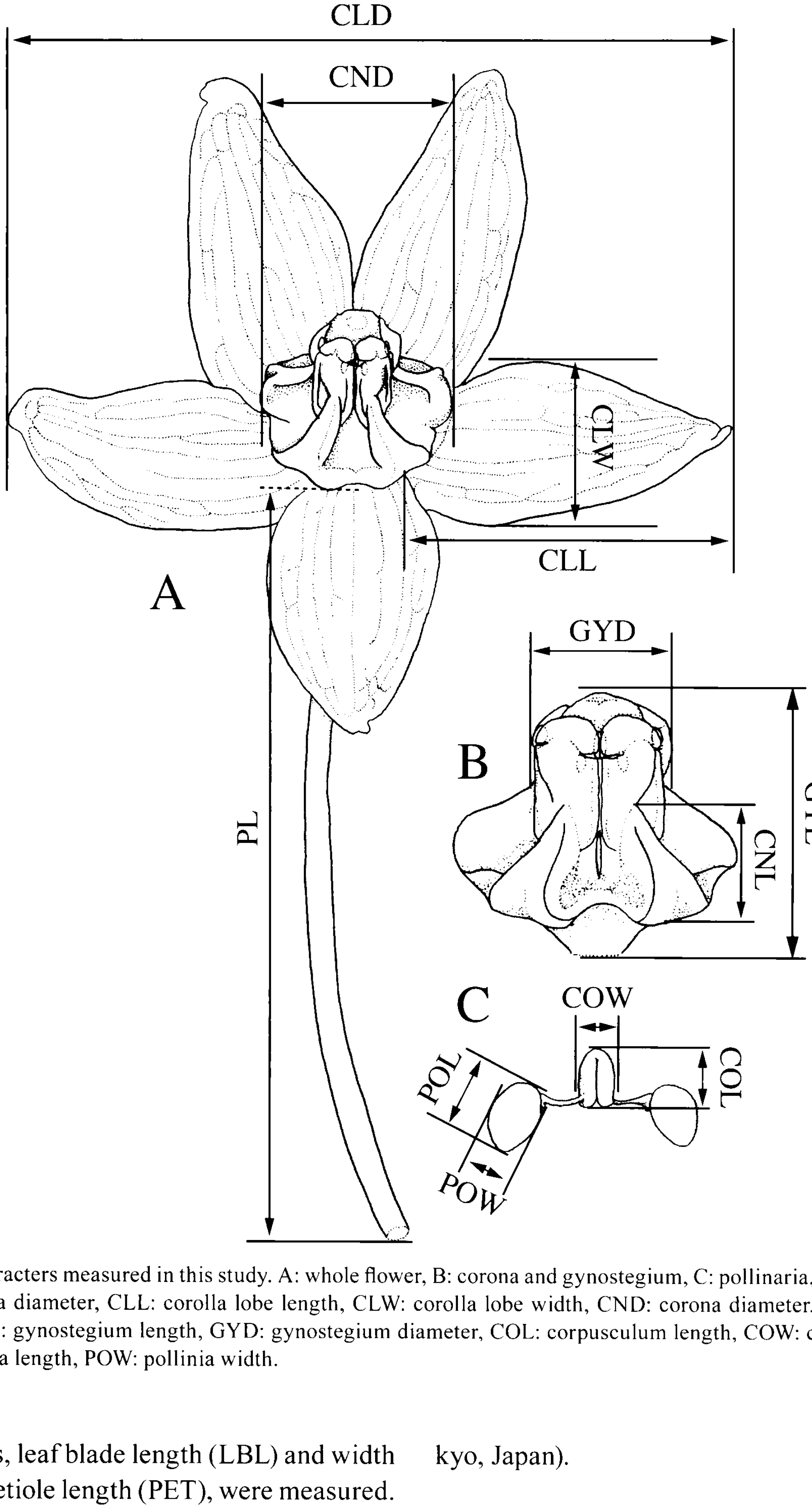

FIG. 2. Floral characters measured in this study. A: whole flower, B: corona and gynostegium, C: pollinaria, PL: pedicel length, CLD: corolla diameter, CLL: corolla lobe length, CLW: corolla lobe width, CND: corona diameter, CNL: corona lobe length, GYL: gynostegium length, GYD: gynostegium diameter, COL: corpusculum length, COW: corpusculum width, POL: pollinia length, POW: pollinia width.

characteristics, leaf blade length (LBL) and width (LBW), and petiole length (PET), were measured.

Principal component analysis (PCA) was performed on 12 floral and three leaf characteristics to summarize the overall tendencies of the morphological variation using Excel-Tokei 2000 (Social Survey Research Information Co., Ltd., Tokyo, Japan).

Allozyme electrophoresis

For each plant, 100 mg of fresh leaf tissue was homogenized in 1mL of an extraction buffer comprising 93 mM Tris-HCl (pH 7.5), 23.4% glycerol, 0.5% (v/v) Tween 80, 2.8 mM dithiothreitol, and 0.5% 2-mercaptoethanol (Uchida *et al.* 1991). The homogenates were centrifuged at 20,000 x g at 4°C for 15 min. The resulting supernatants were used as crude enzyme extracts. Polyacrylamide vertical slab gel electrophoresis was carried out according to procedures described by Davis (1964) and Ornstein (1964). 10 μL crude enzyme extracts were analyzed by polyacrylamide gel electrophoresis. Six enzyme systems were examined: aspartate amino-transferase (AAT, EC 2.6.1.1), colorimetric esterase (EST, EC 3.1.1), leucine aminopeptidase (LAP, EC 3.4.11.1), malate dehydrogenase (MDH, EC 1.1.1.40), phosphoglucoisomerase (PGI, EC 5.3.1.9), and shikimate dehydrogenase (SKDH, EC 1.1.1.25). The staining protocols used were those of Tsumura *et al.* (1990).

The following indices were used to quantify genetic diversity within each population: the proportion of polymorphic loci (P) at the 95% criterion, the number of alleles per locus (A), the expected heterozygosity (h), and the inbreeding coefficient (F_{IS}). As in Hamrick and Godt (1989), the loci that were polymorphic in at least one population were treated as polymorphic at the species level. The significance of deviation of F_{IS} from zero was evaluated using a permutation test. To calculate A, h, and F_{IS}, we used FSTAT 2.9.3 (Goudet 2001).

The genetic differentiation among the populations of *Vincetoxicum katoi* and *V. yamanakae* was determined using F_{ST} of Weir & Cockerham (1984). The significance of pair-wise F_{ST} values was tested by applying the adjusted sequential Bonferroni correction based on 1,000 permutations. All estimates of F_{ST} and their tests of significance were performed using FSTAT 2.9.3 (Goudet 2001). To access the overall population genetic differentiation tendency, the gene diversity statistics, corrected for small sample size errors, were also estimated (Nei & Chesser 1983). The genetic relationships among the *V. katoi* and *V. yamanakae* populations were evaluated by generating a neighbor-joining tree based on the unbiased genetic distance (Nei 1978) using POPTREE2 (Takezaki *et al.* 2010). The significance of the topology was estimated with 1,000 bootstrap replicates. To assess components of genetic variation within and between geographical regions, analyses of molecular variance (AMOVAs; Excoffier *et al.* 1992) with a significances test of 999 random permutations were performed using GenAlEx 6 (Peakall & Smouse 2006).

Results

Morphology

The morphological characteristics of the eight populations analyzed are summarized in Appendix 1. The variances accounted for by PC1 and PC2 were 46.2% and 11.0% of the morphometric data, respectively. The principal component loadings on the first and second components are listed in Table 2. The plots of samples on PC1 and PC2 are shown in Fig. 3. The PC1 represents well the total size of flowers and leaves. For the PC2, whereas the COL, COW, PL and POW were positively correlated, the CNL, LBL, LBW and PET were negatively correlated. Therefore, samples having large pollinia, longer pedicels, smaller leaves and smaller corona tended to appear in the positive field for PC2. The K3 population clustered separately, although other populations were not clearly separated.

TABLE 2. Principal component loading on the first and second components.

	Principal component	
Character	1	2
PL	0.400	0.623
CLD	0.866	0.037
CLL	0.802	-0.098
CLW	0.793	-0.068
CND	0.887	-0.189
CNL	0.782	-0.248
GYD	0.877	0.151
GYL	0.889	-0.090
COL	0.560	0.368
COW	0.406	0.627
POL	0.726	0.052
POW	0.512	0.241
LBL	0.536	-0.352
LBW	0.324	-0.225
PET	0.349	-0.580

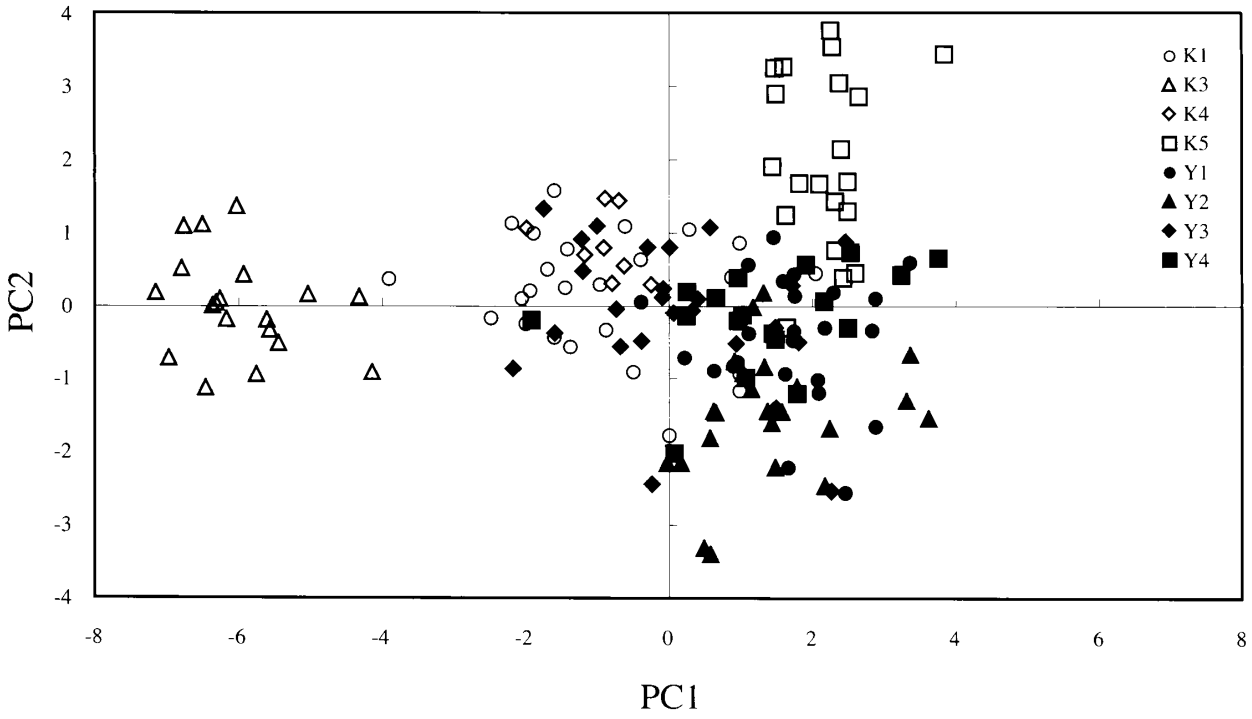

FIG. 3. Plot of principal component analysis for nine populations in the *V. katoi–V. yamanakae* complex.

Genetic diversity

A total of 10 putative loci were detected: *Aat-1*, *Aat-2*, *Est-1*, *Est-2*, *Lap*, *Mdh-1*, *Mdh-2*, *Pgi-1*, *Pgi-2*, and *Skdh*. However, because the *Aat-2*, *Est-2*, *Mdh-2*, and *Pgi-2* loci were not interpretable in all nine populations examined due to weak staining, they were discarded. Among the six loci scored, *Mdh-1* was monomorphic in all populations. The other five loci were polymorphic in at least one population. Allele frequencies of the six loci examined for each population are shown in Appendix 2. Table 3 shows the *P*, *A*, *h* and F_{IS} values for each population and for the overall population. The percentage of polymorphic loci (*P*) ranged from 50.0% (K4 and Y2) to 83.3% (K1-3, Y1, and Y3). The mean number of alleles (*A*) ranged from 1.67 (K4 and Y2) to 3.67 (K1). The expected heterozygosity (*h*) ranged from 0.169 (K5) to 0.488 (K1). The genetic diversity was highest in the K1 population and lowest in the Y2 population. In none of the nine populations examined did the inbreeding coefficient (F_{IS}) value deviate significantly from zero (Table 3).

TABLE 3. Proportion of polymorphic loci (*P*), mean number of alleles (*A*), expected heterozygosity (*h*), and inbreeding coefficient (F_{IS}) at six loci for the populations examined. All F_{IS} values did not significantly deviate from zero by permutation test.

Population	*P*	*A*	*h*	F_{IS}
K1	83.3	3.67	0.488	0.181
K2	83.3	2.67	0.443	-0.005
K3	83.3	3.00	0.356	0.022
K4	50.0	1.67	0.233	-0.071
K5	66.7	2.00	0.169	0.098
Y1	83.3	2.17	0.283	0.021
Y2	50.0	1.67	0.174	0.009
Y3	83.3	2.83	0.373	0.118
Y4	66.7	2.00	0.300	-0.034
Mean	72.1	2.40	0.313	0.037
Overall	83.3	4.17	0.468	0.119

Genetic structure

Pairwise comparisons of genetic differentiation (F_{ST}) across all six loci in the nine populations examined are shown in Table 4. The F_{ST} values between the populations ranged from 0.042 to 0.766, with an average of 0.337. The K5 population was highly genetically differentiated from the other populations (0.395–0.766). The genetic differentiation between the Kanto–Tokai and

TABLE 4. Pair-wise F_{ST} among nine populations of *V. katoi–V. yamanakae* complex. All values significantly departed from zero.

Population	K2	K3	K4	K5	Y1	Y2	Y3	Y4
K1	0.090	0.152	0.271	0.395	0.323	0.468	0.284	0.327
K2		0.042	0.271	0.451	0.292	0.529	0.291	0.280
K3			0.322	0.464	0.343	0.525	0.308	0.273
K4				0.640	0.085#	0.445	0.082#	0.175
K5					0.597	0.766	0.495	0.596
Y1						0.336	0.130	0.215
Y2							0.243	0.428
Y3								0.206

#indicates non significant after sequential Bonferroni correction.

Kii–Shikoku regions was also large (F_{ST} = 0.271–0.529). The genetic differentiation among the Kii–Shikoku populations was higher than in the Kanto–Tokai populations, with the exception of K4–Y1, and K4–Y3 (not significant after Bonfferoni correction). The total genetic diversity (H_T) and coefficient of gene differentiation (G_{ST}) for all populations of *Vincetoxicum katoi* and *V. yamanakae* was 0.488 and 0.356, respectively. The latter value indicates that 64.4% of the total genetic variation was shared by all nine populations and the remaining 35.6% was partitioned within each population.

A neighbor-joining tree based on Nei's (1978) unbiased genetic distance for each population is shown in Fig. 4. The nine populations examined were largely divided into two clusters. One included the K1, 2, 3, and 5 populations, and the other comprised the Y1–4 and K4 populations.

Due to the NJ population clustering result, we performed an AMOVA analysis of the nine populations after division into three regions (K1–3, K5, Y1–3 with K4). The genetic divergence between the three regions was highly significant

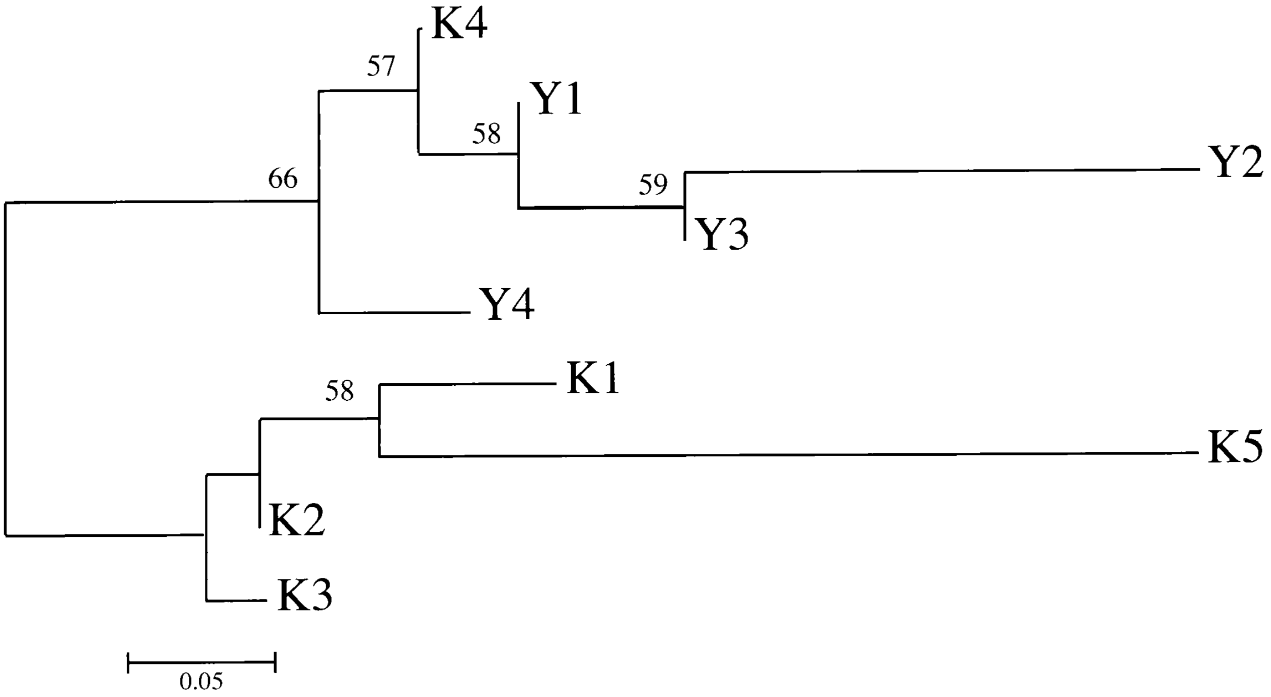

FIG. 4. Phenogram using neighbor-joining method based on unbiased genetic distance (Nei 1978) for nine populations in the *V. katoi–V. yamanakae* complex.

TABLE 5. Results of analysis of molecular variance (AMOVA) for populations of the *V. katoi–V. yamanakae* complex in three regions (Kanto-Tokai, middle Kinki, and Kii-Shikoku).

Source of variation	df	Sum of squares	variance components	% of variation	Phi	*P*
Among regions	1	284.432	1.825	36%	$PhiRT = 0.364$	< 0.001
Among populations within regions	7	151.340	0.969	19%	$PhiPR = 0.304$	< 0.001
Within populatios	218	483.778	2.219	44%	$PhiPT = 0.557$	< 0.001
Total	226	919.551	5.013	100%		

($PhiRT = 0.282$, $P < 0.001$) (Table 5).

Discussion

Taxonomic status of V. katoi *and* V. yamanakae

Vincetoxicum katoi had been previously distinguished from *V. yamanakae* by its smaller corolla and corona (Yamazaki 1993). In contrast, our quantitative analysis of floral morphology did not support the discontinuity of these species. Only the K3 population was separated from the other populations. Certainly, some herbarium specimens collected in the Tokai region exhibit small flowers (corolla lobes 2.5–4 mm in length) and leaves in good agreement with the original description of *V. katoi* (Ohwi 1943). Other populations in the *V. katoi* distribution area, however, were not morphologically distinguishable from *V. yamanakae*. Furthermore, because the Kii peninsula population clustered with populations from Shikoku, the neighbor-joining tree based on six allozyme loci did not support the recognition of two species. Thus, it is appropriate to treat *V. yamanakae* as a synonym of *V. katoi*.

Genetic diversity

Despite their highly restricted and disjunct distribution, our allozyme analysis revealed that members of the *V. katoi–V. yamanakae* complex maintain high genetic diversity at both the species and population levels. At the population level, the three genetic diversity parameters of *V. katoi–V. yamanakae* complex ($P = 72.1$, $A = 2.40$, $h = 0.313$) were higher than those of endemic species ($P = 41.3$, $A = 1.80$, $h = 0.069$) and perennial herbs ($P = 41.3$, $A = 1.79$, $h = 0.012$) (Hamrick & Godt 1989). When compared with a highly outcrossing North American species of *Asclepias* (*A. exaltata* L.: $A = 2.36$, $h = 0.182$, Broyles & Wyatt (1993)), members of the *V. katoi–V. yamanakae* complex exhibit higher genetic diversity. In the nine populations examined, inbreeding coefficient values did not significantly deviate from zero, indicating that outcrossing predominates (Table 1). Outcrossing species tend to retain greater genetic variation than selfing species (Loveless & Hamrick 1984). Therefore, outcrossing may contribute to the maintenance of high genetic diversity in the *V. katoi–V. yamanakae* complex. Another possible factor in the high genetic diversity is species of ancient origin and with a long evolutionary history (e.g., *Kirengeshoma*: Zhang *et al.* 2006, Chang *et al.* 2007). Diversification in the Japanese endemic species of *Vincetoxicum*, however, is thought to have occurred relatively recently due to the paucity of cpDNA and nrDNA variability (Yamashiro *et al.* 2004, 2008). The high genetic diversity in *V. katoi–V. yamanakae* complex may be the result of ancestral polymorphisms. Because the habitat in which they now occurred may have been altered to *Cryptomeria japonica* plantations more or less 50 years ago, they might still maintain high genetic diversity.

Genetic differentiation

In general, outcrossing species are expected to exhibit limited genetic differentiation among populations due to increased gene flow (Loveless & Hamrick 1984). The coefficient of genetic differentiation (G_{ST}) among the nine populations of the *V. katoi–V. yamanakae* complex was 0.356, much higher than the mean for endemic plants (G_{ST} = 0.248), short-lived perennial herbs (G_{ST} = 0.233), and outcrossing animal-pollinated plants (G_{ST} = 0.197) (Hamrick & Godt 1989). The genetic differentiation among the populations of the *V. katoi–V. yamanakae* complex greatly exceeded the genetic differentiation in the species of *Asclepias* in North America (Broyles & Wyatt 1993, Edwards & Wyatt 1994, Broyles 1998). Species of *Asclepias* are mainly pollinated by bumble-bees, leading to pollen-mediated long-distance gene flow, and therefore low population genetic differentiation (Broyles & Wyatt 1993). In contrast, pollination by fungus gnats was observed in the Kanto and Tokai populations of *V. katoi–V. yamanakae* complex (Yamashiro *et al.* 2008), suggesting that pollen-mediated gene flow is restricted in the *V. katoi–V. yamanakae* complex. Although these species have seeds with hairs (coma) that are likely wind-dispersed, they predominantly inhabit the closed understory of *Cryptomeria japonica* plantations, making their dispersal efficiency lower than in congeneric open-habitat species. In fact, the distribution of plants in the *V. katoi–V. yamanakae* complex is limited even on the same mountain; no other populations were found in neighboring valleys or in neighboring *Cryptomeria japonica* plantations. Colonization by seed dispersal may also be limited in these plants.

The nine populations in the *V. katoi–V. yamanakae* complex were genetically divisible into three regions: Kanto–Tokai, middle Kinki (Hyogo), and Kii–Shikoku. The genetic differentiation among these regions was relatively large (*PhiRT* = 0.364 in AMOVA).

The current regional genetic differentiation and distribution of plants in the *V. katoi–V. yamanakae* complex may be due to historical factors. At the last glacial maximum (25,000–15,000 years ago), Honshu and Shikoku were covered with boreal coniferous forests. Evergreen plants in Japan are presumed to have reestablished their postglacial range expansion from southern coastal refugia during the last 12,000 years (Tsukada 1984). The Pacific coast is thought to have been a refugium for evergreen forests during the most recent glacial maximum (Hattori 2002). In *V. katoi–V. yamanakae* complex, the Kanto–Tokai and Kii–Shikoku, regions are located in close proximity and appear to have been glacial refugia, based on palynological analysis (e.g., Muroto, Ashizuri, Shionomisaki, Boso; Hattori 2002). The population differentiation within the Kanto–Tokai region (F_{ST} = 0.042–0.152) is smaller than in the Kii–Shikoku region (0.130–0.428), suggesting that the Kanto–Tokai populations may have expanded from a single refugium, whereas the Kii–Shikoku populations were derived from multiple refugia.

In particular, the K5 population is located outside of known evergreen forest refugia. The post-glacial migration route on the Setouchi Sea side of the Kinki district was considered a route from Shikoku or the Kii peninsula (Hattori 2002, Aoki *et al.* 2004). Although the K5 population is genetically similar to populations in the Kanto–Tokai region than those in the Shikoku–Kii regions, long-distance dispersal of the Kanto–Tokai population to middle Kinki (Hyogo) is unlikely to have occurred, based on the large genetic differences between these regions (F_{ST} = 0.395–0.464). Such a large genetic difference may indicate the existence of refugia for the *V. katoi–V. yamanakae* complex around the K5 population, although evergreen forests are unlikely to have existed nearby. Our results suggest that not all species currently growing in the evergreen forest zone may have migrated in accompaniment with evergreen forest species. The K5 population may have survived during the glacial period within a cooler vegetative zone, such as in a temperate forest, while the Pacific-side populations survived in coastal warm-temperature refugia.

Taxonomy

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APPENDIX 1. Mean ± standard deviation of 12 floral and three leaf characters in eight populations of *V. katoi–V. yamanakae* complex. Abbreviations of characters are given in the text.

Population Code	PL (mm)	CLD (mm)	CLL (mm)	CLW (mm)	CND (mm)	CNL (mm)	GYD (mm)	GYL (mm)
K1	10.3 ± 2.6	11.4 ± 1.3	4.7 ± 0.5	2.9 ± 0.4	3.0 ± 0.3	1.4 ± 0.2	1.6 ± 0.1	2.5 ± 0.2
K3	7.8 ± 2.4	9.7 ± 1.4	4.4 ± 0.7	2.1 ± 0.3	1.8 ± 0.1	0.7 ± 0.1	1.3 ± 0.1	1.5 ± 0.1
K4	15.7 ± 3.1	13.2 ± 1.0	5.8 ± 0.4	3.3 ± 0.1	3.1 ± 0.2	1.3 ± 0.1	1.6 ± 0.1	2.3 ± 0.1
K5	21.4 ± 5.3	15.3 ± 1.3	6.8 ± 0.5	4.2 ± 0.3	3.3 ± 0.2	1.3 ± 0.1	1.8 ± 0.1	2.6 ± 0.2
Y1	12.1 + 3.4	14.0 ± 1.5	7.2 ± 1.0	4.5 ± 0.9	3.9 ± 0.3	1.6 ± 0.3	1.7 ± 0.1	2.6 ± 0.2
Y2	8.5 ± 3.7	14.8 ± 1.3	7.2 ± 0.9	4.0 ± 0.6	3.9 ± 0.3	1.6 ± 0.2	1.6 ± 0.1	2.7 ± 0.2
Y3	9.9 ± 2.2	13.9 ± 1.5	6.2 ± 0.7	3.3 ± 0.3	3.1 ± 0.3	1.2 ± 0.1	2.1 ± 2.7	2.4 ± 0.2
Y4	12.7 ± 4.5	14.6 ± 1.8	7.1 ± 1.1	3.5 ± 0.4	3.6 ± 0.3	1.6 ± 0.2	1.8 ± 0.1	2.6 ± 0.4

APPENDIX 1. Continued.

Population Code	COL (mm)	COW (mm)	POL (mm)	POW (mm)	LBL (mm)	LBW (mm)	PET (mm)
K1	0.30 ± 0.03	0.15 ± 0.02	0.26 ± 0.02	0.16 ± 0.01	14.1 ± 2.8	6.1 ± 1.2	13.4 ± 3.4
K3	0.23 ± 0.02	0.12 ± 0.01	0.20 ± 0.02	0.14 ± 0.01	11.0 ± 2.8	4.2 ± 0.9	9.6 ± 2.7
K4	0.25 ± 0.01	0.14 ± 0.01	0.26 ± 0.02	0.17 ± 0.01	11.3 ± 1.6	4.5 ± 1.1	10.9 ± 1.5
K5	0.32 ± 0.04	0.17 ± 0.02	0.28 ± 0.03	0.18 ± 0.01	15.4 ± 1.5	5.9 ± 0.8	12.2 ± 4.5
Y1	0.28 ± 0.02	0.14 ± 0.01	0.27 ± 0.03	0.16 ± 0.02	14.0 ± 2.2	5.3 ± 0.8	15.0 ± 5.0
Y2	0.27 ± 0.02	0.13 ± 0.01	0.27 ± 0.03	0.17 ± 0.02	15.9 ± 1.7	5.8 ± 0.9	15.8 ± 3.0
Y3	0.26 ± 0.02	0.14 ± 0.02	0.29 ± 0.03	0.18 ± 0.02	14.7 ± 2.7	6.9 ± 1.3	11.5 ± 4.5
Y4	0.29 ± 0.02	0.13 ± 0.01	0.29 ± 0.02	0.17 ± 0.03	14.0 ± 2.2	4.3 ± 0.6	14.4 ± 2.9

APPENDIX 2. Allele frequencies for six loci in the *Vincetoxicum katoi-yamanakae* complex.

		K1	K2	K3	K4	K5	Y1	Y2	Y3	Y4
Aat-1	a	0.208	-	0.083	-	0.060	0.120	0.890	0.375	0.173
	b	-	0.154	0.150	-	0.040	-	-	0.038	-
	c	0.542	0.769	0.750	0.700	0.900	0.880	0.110	0.588	0.827
	d	0.250	0.077	0.017	0.300	-	-	-	-	-
Est-1	a	-	-	-	0.300	-	0.160	-	0.138	0.269
	b	-	-	-	0.700	-	0.840	1.000	0.862	0.731
	c	0.688	0.615	0.661	-	-	-	-	-	-
	d	0.313	0.385	0.339	-	1.000	-	-	-	-
Lap	a	0.042	-	0.033	-	0.040	-	-	0.009	-
	b	0.604	0.538	0.617	0.800	0.960	0.480	0.350	0.824	0.385
	c	0.146	0.192	0.217	-	-	0.520	0.440	0.111	-
	d	0.083	0.115	0.133	0.200	-	-	0.210	0.056	0.615
	e	0.125	0.154	-	-	-	-	-	-	-
Mdh-1	a	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
Pgi-1	a	0.188	0.192	-	-	0.880	0.080	-	0.037	-
	b	0.563	0.808	0.823	0.700	0.120	0.920	1.000	0.935	0.981
	c	0.146	-	0.081	-	-	-	-	-	-
	d	0.104	-	0.097	0.300	-	-	-	0.028	0.019
Skd	a	0.021	0.154	-	-	-	-	-	-	-
	b	0.125	-	0.034	0.600	0.520	0.320	0.110	0.236	0.152
	c	0.146	0.231	-	-	-	0.020	-	-	-
	d	0.063	0.308	0.724	0.100	-	0.080	-	0.179	0.261
	e	0.083	0.308	0.241	0.300	0.460	0.580	0.890	0.585	0.587
	f	0.542	-	-	-	0.020	-	-	-	-
	g	0.021	-	-	-	-	-	-	-	-